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Designing a HER2/*neu* promoter to drive α 1,3galactosyltransferase expression for targeted anti- α Gal antibody-mediated tumor cell killing

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Abstract

Introduction Our goal was to specifically render tumor cells susceptible to natural cytolytic anti- α Gal antibodies by using a murine α 1,3galactosyltransferase (m α GalT) transgene driven by a designed form of HER2/*neu* promoter (p*Neu*), the transcription of which is frequently observed to be above basal in breast tumors. Indeed, the α GalT activity that promotes Gal α 1,3Gal β 1,4GlcNAc-R (α Gal) epitope expression has been mutationally disrupted during the course of evolution, starting from Old World primates, and this has led to the counter-production of large amounts of cytotoxic anti- α Gal antibodies in recent primates, including man.

Method Expression of the endogenous *c-erbB-2* gene was investigated in various cell lines by northern blotting. A m α GalT cDNA was constructed into pcDNA3 vector downstream of the original CMV promoter (pCMV/m α GalT) and various forms of p*Neu* were prepared by PCR amplification and inserted in the pCMV/m α GalT construct upstream of the m α GalT cDNA, in the place of the CMV promoter. These constructs were transferred

into HEK-293 control and breast tumor cell lines. Stably transfected cells were analyzed by northern blotting for their expression of α GalT and *c-erbB-2*, and by flow cytometry for their binding with fluorescein isothiocyanate-conjugated *Griffonia simplicifolia*/isolectin B4.

Results We show that expression of the m α GalT was up- or down-modulated according to the level of endogenous p*Neu* activity and the particular form of constructed p*Neu*. Among several constructs, two particular forms of the promoter, p*Neu*250 containing the CCAAT box and the PEA3 motif adjacent to the TATAA box, and p*Neu*664, which has three additional PEA3 motifs upstream of the CCAAT box, were found to promote differential α GalT expression.

Conclusion Our results strengthen current concepts about the crucial role played by the proximal PEA3 motif of p*Neu*, and may represent a novel therapeutic approach for the development of targeted transgene expression.

Introduction

The enzyme α 1,3galactosyltransferase (α GalT) is responsible for the synthesis of galactose- α 1,3galactose- β 1,4N-acetylglucosamine-R (α Gal) epitopes in all mammals except Old World primates [1]. Highly expressed in nonprimate mammals, prosimians and New World monkeys, this glycosyltransferase has been mutationally inactivated during the course of evolution, starting from Old World primates [2]. We have previously shown that, in human cells, transcription of the α GalT gene is interrupted by the presence of a strong stop signal in exon 7,

which leads to a chimeric mRNA comprising the first four coding exons and part of intron VII, but lacking the last two exons corresponding to the catalytic domain of the enzyme [3]. As a consequence, and given the broad circulation of α Gal carbohydrate antigens, humans, apes and Old World monkeys produce large amounts of anti- α Gal antibodies, which represent approximately 1% of total IgG in humans [4]. These antibodies are responsible for the hyperacute rejection of xenografts and thus prevent trials on transplantation of pig organs to humans [5,6]. Conversely, they represent a potential constitutive tool for therapeutic applications because their highly efficient

ad5 = adenovirus type 5; AP-2 = activator protein-2; bp = base pair; FCS = fetal calf serum; FITC-GS-I-B4 = fluorescein isothiocyanate-conjugated *Griffonia simplicifolia*/isolectin B4; α Gal = galactose- α 1,3galactose- β 1,4N-acetylglucosamine-R; α GalT = α 1,3galactosyltransferase; GAPDH = glyceraldehyde 3-phosphate-dehydrogenase; LTR = long terminal repeat; m α GalT = murine α GalT; NF- κ B = nuclear factor κ B; pCMV = CMV promoter; PCR = polymerase chain reaction; p*Neu* = HER2/*neu* promoter; VSV-G = vesicular stomatitis viral G glycoprotein.

cytolytic activity could be directed against pathological cells transgenically modified to express α Gal epitopes [7-10].

Gene therapy based on the induction of cytotoxicity generally makes use of transgenes that encode prodrug activating enzymes [11]. In the case of anti- α Gal-induced cytotoxicity, no chemical drug is needed to obtain the effect of α GalT because natural circulating anti- α Gal antibodies are sufficient to promote cell lysis via complement activation. One common problem with gene therapy is target cell selectivity. A frequent solution to this is the use of tissue-specific or tumor-activated promoters to drive expression of the transgene [12,13]. Human c-erbB-2 (synonyms erbB2, HER2/*neu*), a member of the *erbB* family that is overexpressed in about one third of breast tumors and in a variety of other tumors, is often correlated with a poor prognosis [14-16]. This gene is normally expressed at a low level in a variety of human embryonic and adult epithelial and hematopoietic cells [17,18]. The high overexpression of c-erbB-2 in tumor cells [19] results from multiple mechanisms, including gene amplification and transcription modulation [20-22]. c-erbB-2 is a 185 kDa transmembrane tyrosine kinase receptor related to the epidermal growth factor receptor that functions as a growth factor receptor to regulate cell growth and transformation [23-25]. Regulation of the c-erbB-2 promoter (*pNeu*) has been extensively investigated in a domain located within the 700 bp region upstream of its transcription start site. A -213/-87 fragment relative to the gene's transcription start site contains the minimal promoter region able to drive preferential transgene expression in breast cancer cells [26].

The present study was designed to obtain targeted expression of α Gal epitopes by human breast cancer cells in order to render them susceptible to anti- α Gal antibody-induced cell death. For this purpose, we used a murine α GalT ($m\alpha$ GalT) transgene driven by a form of *pNeu* designed to be significantly activated in breast tumor cells.

Materials and methods

Cells and reagents

The cell line HEK-293 (ATCC CRL-1573) and the human breast cancer cell lines MCF-7 (ATCC HTB-22), SK-BR-3 (ATCC HTB-30), MDA-MB-231 (ATCC HTB-26), and MDA-MB-453 (ATCC HTB-131) were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Rockville, MD, USA) supplemented with 10% fetal calf serum (FCS; BioWhittaker, Rockland, ME, USA). Fluorescein isothiocyanate-conjugated *Griffonia simplicifolia* I isolectin B4 (FITC-GS-I-B4), which recognizes a terminal galactosyl residue in an α linkage, was purchased from EY Laboratories (San Matteo, CA, USA). The rabbit complement ORAX 07 was from Dade Behring (Paris, France).

Murine α GalT constructions in a plasmid vector and transfection

The $m\alpha$ GalT cDNA was kindly provided by Uri Galili and cloned within *HindIII/XbaI* sites into pcDNA3 vector (Invitrogen, Cergy-Pontoise, France), downstream of the CMV early promoter (pCMV) or various truncated forms of the HER2/*neu* promoter (*pNeu*) obtained by PCR on genomic DNA extracted from human CEM cells (ATCC CCL-119) using the following primer sets: 5'-GGGGGTCCTGGAAGCCACAAG-3' and 5'-GTGCTCACTGCGGCTCCGGCC-3' for *pNeu664* (-488/+176); 5'-TCGCGAGCAGGCAACCCAGGCGTCCCG-3' and 5'-AAGCTTCTCCCCTGGTTTCTCCGGTCCCAA-3' for *pNeu250* (-216/+34); 5'-TCGCGAGCAGGCAACCCAGGCGTCCCG-3' and 5'-CCAAAAGCTTGTGCTCACTGCGGCTCCGGCC-3' for *pNeu392* (-216/+176); 5'-GGAAATCGCGAAGGAAGTATAAGAATGAAG-3' and 5'-CCAAAAGCTTGTGCTCACTGCGGCTCCGGCC-3' for *pNeu209* (-33/+176). All *pNeu* derivative forms were constructed within *NruI/HindIII* sites upstream of *mGalT* cDNA in the place of pCMV.

Cells were transfected in six-well plates using FuGENE 6 transfection reagent (Roche Diagnostics, Meylan, France), as recommended by the manufacturer. Stably transfected cells were selected by G418 resistance.

Murine α GalT constructions in a retroviral vector

To overcome the poor efficiency of classic methods of transfection in MDA-MB-231 cells, a retroviral vector system was developed. The undesirable promoting activity of the 5' long terminal repeat (LTR) was avoided by constructing the cassette *pNeu250/m α GalT* in a self-inactivating murine retroviral vector (pcPM Δ U3) that had been prepared by removing nearly the entire U3 region of the 3' LTR (Lefebvre JC and March D, manuscript in preparation). Making use of this strategy, described in [27], the U3 deletion is transferred to the 5' LTR during the first retrotranscription of the retroviral construct, and further results in the transcriptional inactivation of the provirus in the infected cells. In addition, the cassette *pNeu250/m α GalT* was oriented in the opposite direction (3' to 5') to the LTR so as to completely rule out any residual viral promoter activity. To obtain retroviral particles pseudotyped with a vesicular stomatitis viral G glycoprotein (VSV-G), the plasmid construct was co-transfected in GP2-293 packaging cells with a pVSV-G vector (both from Clontech, BD Biosciences, Le Pont de Claix, France). Supernatants were harvested 48 h post-transfection and filtered (membrane pore size = 0.45 μ m). VSV-G pseudotyped particles were concentrated by ultracentrifugation. Infected cells were seeded in 24-well plates (BD Falcon, Le Pont de Claix, France) and stably transduced subclones were selected by antibiotic resistance. Expression of *m α GalT* was evaluated using GS-I-B4 reactivity.

Flow cytometry analysis

Phenotypic analyses were performed using FITC-GS-I-B4, as previously described [28]. Stained cells were analyzed on a FACScan cytometer (Becton Dickinson, San Jose, CA, USA).

Northern blot analysis

Total RNAs were isolated using RNA Now reagent (Biogentex Inc., Seabrook, TX, USA), according to the manufacturer's instructions, and poly(A)-rich RNAs were selected as described elsewhere [29]. Poly(A)-rich RNAs (3 µg) were electrophoresed on denaturing 1.2% agarose gel and transferred in 20 × NaCl/Citrate onto a Hybond-N+ nylon membrane (Amersham-Biosciences, Saclay, France). Membranes were probed overnight at 42°C with [α - 32 P]-random-labeled m α GalT or *c-erbB-2* cDNA and washed according to standard procedures. The m α GalT probe was excised from the pcDNA3 construct and the *c-erbB-2* probe was obtained by PCR amplification on genomic DNA from SK-BR-3 cells with the primer set 5'-CCAGGAGGTGCAGGGCTACG-3' and 5'-ATCCTCAGAACTCTCTCCCC-3'. Membranes were exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, USA). Detection of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was used as an internal control.

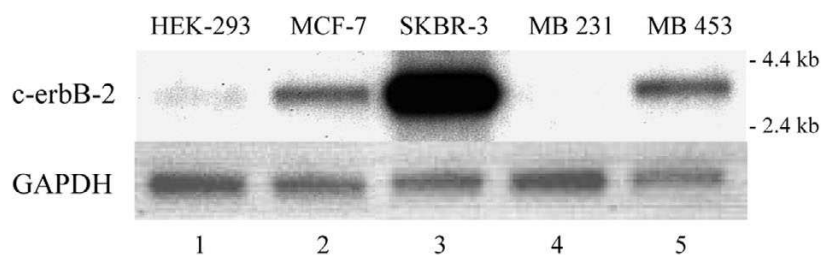
Cytotoxicity assay

Parental and transfected HEK-293, MCF-7, or SK-BR-3 cells were distributed in 96-well plates (5.10⁴ cells/well) and incubated for 1 h after addition of 10 µl of various human sera, in triplicate. Rabbit complement (20 µl) was then added and plates were incubated for 1 h. Cell death was evaluated by the trypan blue vital dye exclusion method. The percentage of killed cells was evaluated by comparison of the number of blue-stained cells in the reaction and control wells.

Cell proliferation assay

After cell incubation with human sera and rabbit complement as above, XTT reagent from the Cell Proliferation Kit II (Roche Diagnostics, Meylan, France) was added, according to the manufacturer's instructions, and the cells were re-incubated at 37°C for 2 h. Formazan formation was measured at 490 nm.

Figure 1



Expression of the *c-erbB-2* gene in various cell lines. Total RNAs from HEK-293 and human breast cancer cell lines MCF-7, SK-BR-3, MDA-MB-231 and MDA-MB-453 were probed with *c-erbB-2* cDNA and thereafter with glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) cDNA as a control.

Statistical analysis

Statistical comparison of mean values was performed with a one-way analysis of variance (ANOVA).

Results

Expression of the *c-erbB-2* gene in various cell lines

Expression of the endogenous *c-erbB-2* gene was investigated in various cell lines by northern blotting. *c-erbB-2* was very weakly expressed in HEK-293 cells and was differentially transcribed in human breast cancer cell lines (Figs 1 and 2C): it was absent in MDA-MB-231, moderately expressed in MCF-7 and MDA-MB-453, and strongly expressed in SK-BR-3 cells. These results are consistent with data published by others [21,30,31]. SK-BR-3 cells are known to overexpress *c-erbB-2* as the result of gene amplification in proportions estimated at up to 13:1 [21]. MCF-7 is a known *HER2/neu* non-amplified cell line [20], but various degrees of gene expression have been reported [30,32-34]. The *HER2/neu* transcription level of the MCF-7 cell line used in our laboratory was notably superior to that of the HEK-293 cells (Fig. 1). Serially passaged in different laboratories, MCF-7 cell lines probably exhibit variable levels of *HER2/neu* transcription. Immortalized by adenovirus type 5 (ad5) [35], HEK-293 cells require continuous ad5 E1A expression to proliferate and avoid senescence [36], and can thus be considered subnormal because they are not tumorigenic [37]. Interestingly, their very weak *HER2/neu* expression done them useful as controls to investigate the activation of various forms of *pNeu* as a function of *HER2/neu* expression in breast tumor cells. Because our goal was to take advantage of differential up-regulation of endogenous *pNeu* to overexpress a suicide transgene that is itself driven by an exogenous *pNeu*, MCF-7 cells without *HER2/neu* gene amplification appeared more suitable than HEK-293 cells. SK-BR-3 cells might provide other information, as detailed hereafter.

Variable activity of m α GalT driven by various forms of *c-erbB-2* promoter in human breast cancer cell lines

Human cells do not express any α GalT activity responsible for α Gal epitope expression that is recognized by the GS-I-B4 lectin. GS-I-B4 binding might thus specifically reveal expression of exogenous α GalT in transgenically modified human cells. A α GalT cDNA was constructed into pcDNA3 vector downstream of the original pCMV (pCMV/ α GalT). In addition, various forms of p*Neu* were prepared by PCR amplification (Fig. 2a): p*Neu*664 (nucleotides -488/+176, relative to the transcription start site of *HER2/neu*), p*Neu*392 (nucleotides -216/+176) and p*Neu*250 (nucleotides -216/+34). These forms were inserted in the pCMV/ α GalT construct upstream of the α GalT cDNA, in the place of the original pCMV (p*Neu*664/ α GalT, p*Neu*392/ α GalT and p*Neu*250/ α GalT). These constructs were transferred into HEK-293 control and breast tumor cell lines. Stably transfected cells were analyzed by northern blotting for their expression of α GalT (Fig. 2b) and c-erbB-2 (Fig. 2c), and by flow cytometry for their binding with FITC-GS-I-B4 (Fig. 3a-c). pCMV and p*Neu*664 promoted noticeable expression of α GalT in both HEK-293 and breast cancer cell lines (Fig. 2b, lanes 2-3, 7-8 and 12-13). In contrast, the shortest form, p*Neu*250, raised α Gal expression to a more specific level in MCF-7 than in HEK-293 cells (Fig. 2b, compare lane 10 to 5). Similar differential results were observed with p*Neu*392 (Fig. 2b, compare lanes 9 and 14 to 4) whereas a complete switch-off was observed with p*Neu*209 (data not shown). These results were verified phenotypically. Elevated α Gal expression was observed in breast tumor and HEK-293 cells with α GalT driven by pCMV or p*Neu*664, while p*Neu*250 promoted α Gal expression only in the breast tumor cells SK-BR-3 and MCF-7 (Fig. 3a). To confirm these results, expression of the cassette p*Neu*250/ α GalT was compared for breast tumor cells expressing and not expressing c-erbB-2. The non-expressing MDA-MB-231 cell line, although appropriate for this purpose, was unfortunately resistant to classic transfection methods. A self-inactivating retroviral vector pseudotyped by a VSV-G glycoprotein was thus used to transfer p*Neu*250/ α GalT. Stably transduced cell lines were subcloned during the course of antibiotic selection. Four to five subclones of each type were analyzed for the expression of α GalT. The MDA-MB-231 cells, which did not express c-erbB-2, showed a very low level of GS-I-B4 reactivity compared to breast tumor cell lines SK-BR-3 and MCF-7, which express c-erbB-2 (Fig. 3c).

Apparently conflicting results show that p*Neu*250 promoted clearly higher α Gal expression in MCF-7 than in SK-BR-3 cells (Fig. 3a,b), while c-erbB-2 was inversely expressed in these two cell lines (Fig. 1, lanes 2 and 3; Fig. 2c, lanes 4 and 7). These data could be explained by the differential mechanisms that sustain *HER2/neu* overexpression, which is regulated at the transcriptional level in MCF-7 cells and is dependent on the existence of multiple gene copies in SK-BR-3 cells. Interestingly, p*Neu*250 promoted a much more specific expression as a function of the differential level of endogenous c-erbB-2

transcription in breast tumor cells (Fig. 1, lanes 2-4) and HEK-293 cells (Fig. 1, lane 1).

Cytolytic activity of anti- α Gal antibodies to transgenically modified breast tumor cells

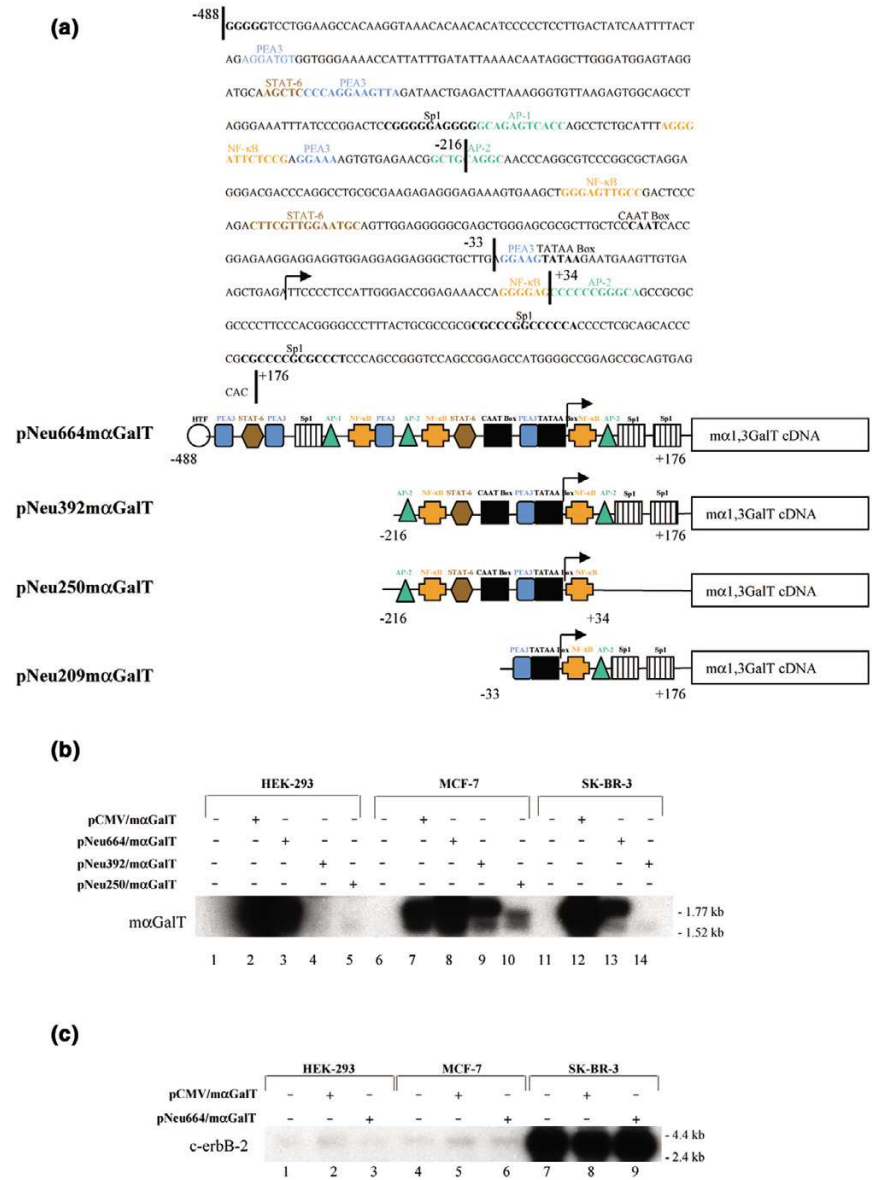
A high fraction of antibodies from human sera bind α Gal epitopes and can efficiently induce the death of cells that exhibit these epitopes via complement activation [4]. Cytotoxicity assays were carried out on HEK-293, MCF-7 and SK-BR-3 cells stably transfected by α GalT cDNA under the control of p*Neu*664 and p*Neu*250, or pCMV used as a control. The susceptibility of transfected cells to natural human anti- α Gal antibodies was verified using a complement-dependent cytotoxicity test (data not shown). Cell death averages were confirmed by an XTT proliferation assay (Fig. 4). All of the cell types transfected with pCMV/ α GalT or p*Neu*664/ α GalT were killed, but to varying degrees (Fig. 4). Interestingly, differential cytolytic activity of antibodies was observed with p*Neu*250/ α GalT, being much greater in MCF-7 and SK-BR-3 cells than in HEK-293 cells (Fig. 4). Here again, α GalT was much more efficiently driven by pCMV or p*Neu*664 in HEK-293 cells than in breast tumor cells. p*Neu*250 gave differential results that were in favor of the tumor cells; it promoted a more significant proportion of death in MCF-7 than in SK-BR-3 cells (Fig. 4) that correlated with their respective levels of α Gal epitope expression (Fig. 3).

Discussion

Efforts to develop anticancer therapies based on suicide transgenes generally focus on prodrug activating enzymes [38] combined with effective targeting of pathological cells. We have been studying α GalT gene expression and the very high efficiency of natural anti- α Gal antibodies in inducing complement-mediated cell killing [39,40] in the field of hyperacute xenograft rejection. We attempted to take advantage of this constitutive immune system to target tumor cells. Several authors have demonstrated the efficacy of natural anti- α Gal antibodies for destruction of tumor cells [7-10]. Indeed, the high numbers of circulating oligosaccharides bearing α Gal epitopes are responsible for constant booster immunizations. This may explain the high plasma level (1% of IgG) of anti- α Gal antibodies in humans [4] and their constant *de novo* synthesis in α GalT knockout mice [41,42]. Natural anti- α Gal antibodies are highly cytotoxic and cytolytic as the result of highly efficient complement activation, and this results in hyperacute rejection of xenografts [2,40]. They have also been shown capable of protecting α GalT-deficient mice against engrafted α Gal⁺ colon cancer cells [9].

The purpose of this study was the targeting of tumor cells known to overexpress c-erbB-2 using a selected form of its promoter p*Neu* to drive an active α GalT. This approach was designed to take advantage of the effective antibodies preexisting in all humans. In a similar study, a derived form of the human telomerase promoter was shown to render human pan-

Figure 2

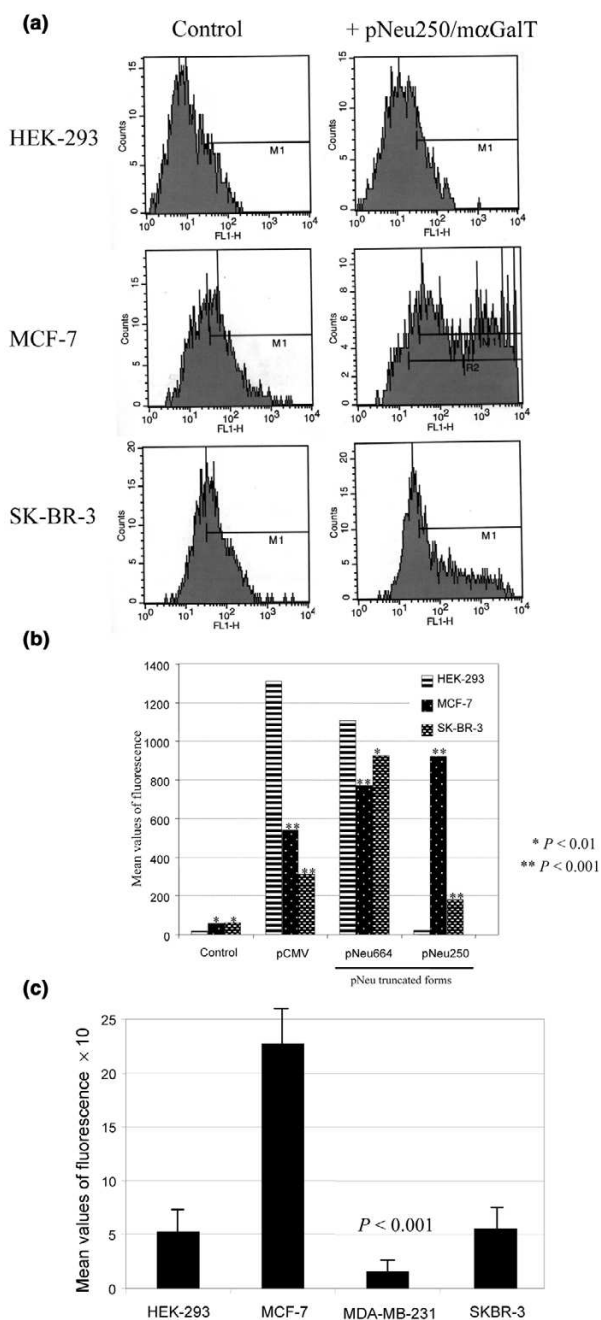


Promoting activity of newly designed forms of *pNeu* in various cell lines differentially expressing *c-erbB-2* **(a)** Schematic representation of various forms of *pNeu* constructed upstream of the murine α 1,3galactosyltransferase (α GalT) cDNA. The transcription start site is indicated by arrows, and the 5' end points of the various forms of *pNeu* by bold bars. **(b)** Northern blot analysis of expression of α GalT driven by the CMV promoter (pCMV) and the various forms of constructed *pNeu*. **(c)** Northern blot analysis of *c-erbB-2* expression in stably transfected HEK-293, MCF-7 and SK-BR-3 cells. Total RNAs were electrophoresed and probed with [α - 32 P]-random-labeled α GalT or *c-erbB-2* cDNA, and thereafter with glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) cDNA as a control.

creatic carcinoma cells susceptible to α Gal/complement-mediated cell killing [43]. We selected *pNeu* because it has been well characterized and is overexpressed in a variety of tumors [16,44]. Because definition of a precise *pNeu* sequence with well-restricted activation in tumor cells remains uncertain, however, we analyzed several forms of this promoter. The shortest form, *pNeu209*, which comprises the only PEA3 motif adjacent to the TATA box plus two SP1 sites and one AP-2 site downstream of the transcription start site, pro-

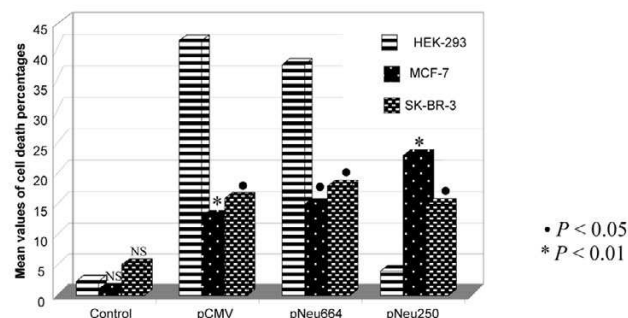
moted very weak α Gal expression. The minimal forms, *pNeu392* and *pNeu250*, were equally capable of selectively inducing α GalT in breast tumor cells compared with HEK-293 cells. We thus concluded that the AP-2 and SP1 motifs downstream of the transcription start site (Fig. 2) were not essential. The noticeable absence of a CCAAT box in *pNeu209* probably explains its disrupted activity because in cells overexpressing *c-erbB-2*, the CCAAT box is up-regulated rather than the TATAA box [45]. Further studies thus focused on

Figure 3



Expression of Gal α 1,3Gal residues at the surface of transduced cells. Cells stably transduced or not with CMV promoter (pCMV)/murine α 1,3galactosyltransferase (α GalT), pNeu664/ α GalT, or pNeu250/ α GalT were labeled with fluorescein isothiocyanate-conjugated *Griffonia simplicifolia*/isolectin B4 (FITC-GS-I-B4) lectin (Gal α 1,3Gal) and analyzed on a FACScan cytometer (counts, cells numbers; FL1-H, fluorescence intensity). **(a)** Binding of FITC-GS-I-B4 to parental and pNeu250/ α GalT transduced HEK-293, MCF-7 and SK-BR-3 cells. Mean values of fluorescence detected by flow cytometry at the surface of stably transduced cells with **(b)** the constructs pNeu664/ α GalT or pNeu250/ α GalT in a plasmid vector (experiments in triplicates), and **(c)** the construct pNeu250/ α GalT in a self-inactivating retroviral vector (experiments were conducted using four to five subclones).

Figure 4



Cytotoxicity assays on HEK-293, MCF-7 and SK-BR-3 cells stably transfected or not with CMV promoter (pCMV)/murine α 1,3galactosyltransferase (α GalT), pNeu664/ α GalT, or pNeu250/ α GalT. Cells were distributed in 96-well plates (5.10⁴/well) and incubated with human sera, after which rabbit complement was added. Cell death was analyzed using an XTT reagent proliferation assay. Results (means of triplicates) are expressed as percentages of cell death, relative to the amount of formazan formation (absorbance evaluated at 490 nm; NS, not significant).

comparing pNeu250 with the longest form, pNeu664. This last form contains several PEA3, NF- κ B, HER2 transcription factor (HTF) and SP1 sites upstream of the minimal pNeu250. The role played by the Ets family and activator protein-2 (AP-2) factors has been extensively studied in breast tumor cells. While the AP-2 binding site was present in both pNeu250 and pNeu664, the main difference between these forms was the presence of three additional PEA3 motifs in pNeu664. Activation of pNeu664 was virtually the same in MCF-7 and SK-BR-3 tumor cells, whereas a marked decrease in pNeu250 activity was observed only in SK-BR-3 cells (Fig. 3), in complete contrast to their high c-erbB-2 expression (Fig. 2c). As discussed above, the striking overexpression of c-erbB-2 in SK-BR-3 cells can be explained by their multiple gene copies. In MCF-7 cells, the differential promoting activity of pNeu250 could be explained by the relative increase in the transcription level compared with HEK-293 cells. In other aspects, in association with c-erbB-2 gene amplification, up-regulation of transcriptional factors that control endogenous pNeu remains possible. Conflicting results have been published on Ets regulation of c-erbB-2, with activation and repression of pNeu by PEA3 factors having been reported [46,47]. The observation that Ets binding leads to a severe bend in DNA could be further support for our findings [46]. When the number of PEA3 binding sites is reduced from four in pNeu664 to one in pNeu250, over-occupation of the single remaining site in pNeu250 might hinder formation of the required DNA conformation rather than favor its reading.

The differential promoting activity of pNeu664 and pNeu250 in HEK-293 cells (Fig. 3) does not appear to be relevant to the transcriptional regulation of c-erbB-2 because this gene is only weakly expressed in these cells (Fig. 1, lane 1). In

contrast, HEK-293 cells continuously express ad5 E1A, which has been shown to target p*Neu* [48] as a repressor of HER2/*neu* overexpression [49], and has been proposed for use in cancer gene therapy [50]. Like the Ets factors expressed in tumor cells, an equal level of E1A in HEK-293 cells might activate p*Neu*664, which contains four PEA3 motifs, and repress p*Neu*250, which has only one.

Our efforts to take advantage of natural cytotoxic anti- α Gal antibodies as a means of destroying breast tumor cells, and to design a promoter specific for these undesirable cells, have to be considered as a preliminary contribution to the field of cancer gene therapy, given that our results have been obtained in cell line culture models. It has been shown that human primary breast tumors can be successfully engrafted into NOD/SCID mice and maintained in a growing state for more than 100 days [51]. Moreover, α GalT(-/-) KO mice have been fortunately generated by others [52,53]. So to progress towards a gene therapy application, we are developing a two step procedure in mice. First, the distribution and expression of the transgene p*Neu*250/ α GalT, cloned into the retroviral vector pcPM Δ U3 (see Materials and methods), will be studied in a human breast cancer xenograft model. Various types of human breast tumor differentially expressing HER2/*neu* will be implanted in NOD/SCID mice, and thereafter the transgene will be injected by a variety of methods. Second, α GalT-transduced tumor pieces will be transplanted into immunocompromised α GalT KO mice to evaluate the tumor destroying activity of purified human anti- α Gal antibodies.

Conclusion

Our results show that the association p*Neu*250/ α GalT could be used to target tumor cells overexpressing c-erbB-2, and thus expose them to the cytolytic activity of natural anti- α Gal antibodies. Development of a discriminating *in vivo* system capable of targeting tumor cells according to their level of c-erbB-2 expression could prove beneficial.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

ML performed the various p*Neu* constructs, transduction assays, Northern blot analyses and participated in literature search and critical reading of the manuscript. LO conducted the statistical analysis and participated in cell proliferation assays. VG carried out flow cytometry analysis and contributed in Northern blot analyses. JCL conceived the study and further developments, looked after data interpretation, and wrote the manuscript. All authors read and approved the final manuscript.

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References

- Galili U: **Evolution of a 1,3Galactosyltransferase and of the α -Gal epitope.** In *α -Gal and Anti-Gal: α -1,3-galactosyltransferase, α -Gal epitopes, and the natural anti-Gal antibody* Edited by: Galili U, Avila JL. New York, NY: Kluwer Academic/Plenum Publishers; 1999:1-18.
- Galili U, Shohet SB, Kobrin E, Stults CL, Macher BA: **Man, apes, and Old World monkeys differ from other mammals in the expression of alpha-galactosyl epitopes on nucleated cells.** *J Biol Chem* 1988, **263**:17755-17762.
- Lanteri M, Giordanengo V, Hiraoka N, Fuzibet JG, Auberger P, Fukuda M, Baum LG, Lefebvre JC: **Altered T cell surface glycosylation in HIV type 1 infection results in increased susceptibility to galectin-1-induced cell death.** *Glycobiology* 2003, **13**:909-918.
- Galili U, Avila JL: *α -Gal and Anti-Gal: α -1,3-galactosyltransferase, α -Gal epitopes, and the natural anti-Gal antibody* New York, NY: Kluwer Academic/Plenum Publishers; 1999.
- Hortobagyi GN, Hung MC, Lopez-Berestein G: **A Phase I multicenter study of E1A gene therapy for patients with metastatic breast cancer and epithelial ovarian cancer that overexpresses HER-2/*neu* or epithelial ovarian cancer.** *Hum Gene Ther* 1998, **9**:1775-1798.
- Buhler L, Friedman T, Iacomini J, Cooper DK: **Xenotransplantation - state of the art - update 1999.** *Front Biosci* 1999, **4**:D416-432.
- Jager U, Takeuchi Y, Porter CD: **Sensitization of human cells to lysis by human complement as an approach for cancer gene therapy.** *Adv Exp Med Biol* 1998, **451**:359-363.
- Link CJ Jr, Seregina T, Atchison R, Hall A, Muldoon R, Levy JP: **Eliciting hyperacute xenograft response to treat human cancer: alpha(1,3) galactosyltransferase gene therapy.** *Anticancer Res* 1998, **18**:2301-2308.
- Unfer RC, Hellrung D, Link CJ Jr: **Immunity to the alpha(1,3)galactosyl epitope provides protection in mice challenged with colon cancer cells expressing alpha(1,3)galactosyl-transferase: a novel suicide gene for cancer gene therapy.** *Cancer Res* 2003, **63**:987-993.
- Aubert M, Crotte C, Bernard JP, Lombardo D, Sadoulet MO, Mas E: **Decrease of human pancreatic cancer cell tumorigenicity by alpha1,3galactosyltransferase gene transfer.** *Int J Cancer* 2003, **107**:910-918.
- Dachs GU, Dougherty GJ, Stratford IJ, Chaplin DJ: **Targeting gene therapy to cancer: a review.** *Oncol Res* 1997, **9**:313-325.
- Ring CJ, Blouin P, Martin LA, Hurst HC, Lemoine NR: **Use of transcriptional regulatory elements of the MUC1 and ERBB2 genes to drive tumour-selective expression of a prodrug activating enzyme.** *Gene Ther* 1997, **4**:1045-1052.
- Manley PW, Martiny-Baron G, Schlaeppli JM, Wood JM: **Therapies directed at vascular endothelial growth factor.** *Expert Opin Investig Drugs* 2002, **11**:1715-1736.
- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: **Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene.** *Science* 1987, **235**:177-182.
- Kraus MH, Popescu NC, Amsbaugh SC, King CR: **Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms.** *Embo J* 1987, **6**:605-610.
- Menard S, Pupa SM, Campiglio M, Tagliabue E: **Biologic and therapeutic role of HER2 in cancer.** *Oncogene* 2003, **22**:6570-6578.
- Brennan PJ, Kumagai T, Berezov A, Murali R, Greene MI, Kumogai T: **HER2/*neu*: mechanisms of dimerization/oligomerization.** *Oncogene* 2000, **19**:6093-6101.
- Leone F, Perissinotto E, Cavalloni G, Fonsato V, Bruno S, Surrenti N, Hong D, Capaldi A, Geuna M, Piacibello W, et al.: **Expression of the c-ErbB-2/HER2 proto-oncogene in normal hematopoietic cells.** *J Leukoc Biol* 2003, **74**:593-601.
- Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al.: **Studies of the**

- HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989, **244**:707-712.
20. Li BD, Harlow SP, Budnick RM, Sheedy DL, Stewart CC: **Detection of HER-2/neu oncogene amplification in flow cytometry-sorted breast ductal cells by competitive polymerase chain reaction.** *Cancer* 1994, **73**:2771-2778.
21. Lyon E, Millson A, Lowery MC, Woods R, Wittwer CT: **Quantification of HER2/neu gene amplification by competitive pcr using fluorescent melting curve analysis.** *Clin Chem* 2001, **47**:844-851.
22. Wang SC, Hung MC: **Transcriptional targeting of the HER-2/neu oncogene.** *Drugs Today (Barc)* 2000, **36**:835-843.
23. Coussens L, Yang-Feng TL, Liao YC, Chen E, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Francke U, et al.: **Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene.** *Science* 1985, **230**:1132-1139.
24. Klapper LN, Glathe S, Vaisman N, Hynes NE, Andrews GC, Sela M, Yarden Y: **The ErbB-2/HER2 oncoprotein of human carcinomas may function solely as a shared coreceptor for multiple stroma-derived growth factors.** *Proc Natl Acad Sci USA* 1999, **96**:4995-5000.
25. Holbro T, Civenni G, Hynes NE: **The ErbB receptors and their role in cancer progression.** *Exp Cell Res* 2003, **284**:99-110.
26. Yu L, Kamo S, Tagawa M: **Identification of a minimal c-erbB-2 promoter region that mediates preferential expression of a linked foreign gene in human breast cancer cells.** *Int J Oncol* 2002, **20**:607-610.
27. Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, Anderson WF, Wagner EF, Gilboa E: **Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells.** *Proc Natl Acad Sci USA* 1986, **83**:3194-3198.
28. Lefebvre JC, Giordanengo V, Limouse M, Doglio A, Cucchiari M, Monpoux F, Mariani R, Peyron JF: **Altered glycosylation of leukosialin, CD43, in HIV-1-infected cells of the CEM line.** *J Exp Med* 1994, **180**:1609-1617.
29. Giordanengo V, Bannwarth S, Laffont C, Van-Miegem V, Harduin-Lepers A, Delannoy P, Lefebvre JC: **Cloning and expression of cDNA for a human Gal(b1-3)GalNAc a2,3-sialyltransferase from the CEM-T cell line.** *Eur J Biochem* 1997, **247**:558-566.
30. Wright M, Grim J, Deshane J, Kim M, Strong TV, Siegal GP, Curiel DT: **An intracellular anti-erbB-2 single-chain antibody is specifically cytotoxic to human breast carcinoma cells overexpressing erbB-2.** *Gene Ther* 1997, **4**:317-322.
31. Brodowicz T, Wilschke C, Budinsky AC, Krainer M, Steger GG, Zielinski CC: **Soluble HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells in vitro.** *Int J Cancer* 1997, **73**:875-879.
32. Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB: **Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells.** *Clin Cancer Res* 1996, **2**:1215-1219.
33. Kurokawa H, Lenferink AE, Simpson JF, Pisacane PI, Sliwkowski MX, Forbes JT, Arteaga CL: **Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells.** *Cancer Res* 2000, **60**:5887-5894.
34. Child SJ, Miller MK, Geballe AP: **Cell type-dependent and -independent control of HER-2/neu translation.** *Int J Biochem Cell Biol* 1999, **31**:201-213.
35. Graham FL, Smiley J, Russell WC, Nairn R: **Characteristics of a human cell line transformed by DNA from human adenovirus type 5.** *J Gen Virol* 1977, **36**:59-74.
36. Quinlan MP: **Expression of antisense E1A in 293 cells results in altered cell morphologies and cessation of proliferation.** *Oncogene* 1993, **8**:257-265.
37. Kamei D, Murakami M, Nakatani Y, Ishikawa Y, Ishii T, Kudo I: **Potential role of microsomal prostaglandin E synthase-1 in tumorigenesis.** *J Biol Chem* 2003, **278**:19396-19405.
38. Springer CJ, Niculescu-Duvaz I: **Prodrug-activating systems in suicide gene therapy.** *J Clin Invest* 2000, **105**:1161-1167.
39. Galili U, Rachmilewitz EA, Peleg A, Flechner I: **A unique natural human IgG antibody with anti-alpha-galactosyl specificity.** *J Exp Med* 1984, **160**:1519-1531.
40. Good AH, Cooper DK, Malcolm AJ, Ippolito RM, Koren E, Neethling FA, Ye Y, Zuhdi N, Lamontagne LR: **Identification of carbohydrate structures that bind human antiporcine antibodies: implications for discordant xenografting in humans.** *Transplant Proc* 1992, **24**:559-562.
41. Thall AD, Murphy HS, Lowe JB: **alpha 1,3-Galactosyltransferase-deficient mice produce naturally occurring cytotoxic anti-Gal antibodies.** *Transplant Proc* 1996, **28**:556-557.
42. Salvaris E, Gock H, Han W, Murray-Segal L, Barlow H, Mottram P, Pearse M, Cowan P, Goodman D, d'Apice AJ: **Naturally acquired anti-alpha Gal antibodies in a murine allograft model similar to delayed xenograft rejection.** *Xenotransplantation* 2000, **7**:42-47.
43. Sawada T, Yamada O, Yoshimura N, Hatori K, Fuchinoue S, Terakawa S: **Xenoantigen, an alphaGal epitope-expression construct driven by the hTERT-promoter, specifically kills human pancreatic cancer cell line.** *Cancer Cell Int* 2002, **2**:14-20.
44. Arteaga CL, Chinratanalab W, Carter MB: **Inhibitors of HER2/neu (erbB-2) signal transduction.** *Semin Oncol* 2001, **28**:30-35.
45. Hurst HC: **Update on HER-2 as a target for cancer therapy: the ERBB2 promoter and its exploitation for cancer treatment.** *Breast Cancer Res* 2001, **3**:395-398.
46. Scott GK, Chang CH, Erny KM, Xu F, Fredericks WJ, Rauscher FJ 3rd, Thor AD, Benz CC: **Ets regulation of the erbB2 promoter.** *Oncogene* 2000, **19**:6490-6502.
47. Xing X, Wang SC, Xia W, Zou Y, Shao R, Kwong KY, Yu Z, Zhang S, Miller S, Huang L, et al.: **The ets protein PEA3 suppresses HER-2/neu overexpression and inhibits tumorigenesis.** *Nat Med* 2000, **6**:189-195.
48. Yu D, Suen TC, Yan DH, Chang LS, Hung MC: **Transcriptional repression of the neu protooncogene by the adenovirus 5 E1A gene products.** *Proc Natl Acad Sci USA* 1990, **87**:4499-4503.
49. Chang JY, Xia W, Shao R, Sorgi F, Hortobagyi GN, Huang L, Hung MC: **The tumor suppression activity of E1A in HER-2/neu-overexpressing breast cancer.** *Oncogene* 1997, **14**:561-568.
50. Yu D, Hung MC: **Overexpression of ErbB2 in cancer and ErbB2-targeting strategies.** *Oncogene* 2000, **19**:6115-6121.
51. Beckhove P, Schutz F, Diel IJ, Solomayer EF, Bastert G, Foerster J, Feuerer M, Bai L, Sinn HP, Umansky V, et al.: **Efficient engraftment of human primary breast cancer transplants in nonconditioned NOD/Scid mice.** *Int J Cancer* 2003, **105**:444-453.
52. Thall AD, Maly P, Lowe JB: **Oocyte Gal alpha 1,3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse.** *J Biol Chem* 1995, **270**:21437-21440.
53. Tearle RG, Tange MJ, Zannettino ZL, Katerelos M, Shinkel TA, Van Denderen BJ, Lonie AJ, Lyons I, Nottle MB, Cox T, et al.: **The alpha-1,3-galactosyltransferase knockout mouse. Implications for xenotransplantation.** *Transplantation* 1996, **61**:13-19.